Influence of GSTM1 and GSTT1 polymorphisms on the survival rate of patients with malignant glioma under perillyl alcohol-based therapy

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ABSTRACT. GSTM1 (glutathione S-transferase mu 1) and GSTT1 (glutathione S-transferase theta 1) are critical enzymes for detoxification of endogenous and environmental carcinogens. Constitutive GST gene polymorphisms may be associated with increased risk for cancer development. We made an explorative study of a Brazilian population with malignant glioma to determine whether GSTM1 and GSTT1 genetic polymorphisms influence the response to intranasal administration of perillyl alcohol and the survival rate. Patients were stratified into groups according to clinical presentation, tumor classification, and tumor location. Circulating DNA was extracted from blood plasma or serum, and genotypes were detected by multiplex PCR. The cohort included
95 patients with recurrent malignant glioma included in a Phase I/II clinical trial with perillyl alcohol and 100 matched healthy control subjects. GSTM1 frequency was similar in patients with glioma (44%) and healthy controls (54%), but GSTT1 deletion was found in 11.5% patients, contrasting with 36% in controls. A longer survival rate was associated with a lack of GSTM1 deletion (31 weeks) and a deletion for GSTT1 (28 weeks). A poor survival rate was associated with GSTM1 deletion (23 weeks) and with a lack of a GSTT1 deletion (19 weeks).

A significantly lower frequency of GSTT1 deletion in glioma patients compared to healthy controls indicates that GSTT1 deletion may exert a protective role against gliomagenesis, influence therapeutic response to intranasal perillyl alcohol treatment, and increase overall survival, especially considering tumor topography.

Key words: Glutathione S-transferases; Genetic polymorphism; Glioma; Perillyl alcohol

INTRODUCTION

Gliomas are the most frequent, heterogeneous group of primary malignant tumors in the adult central nervous system, accounting for more than 50% of all brain tumors (Louis et al., 2007; Adamson et al., 2010). Patients with malignant glioma generally have poor prognosis with 5-year survival less than 5% due to tumor aggressiveness and lack of curative treatment (Adamson et al., 2010). In addition, the anatomical location of the lesion in the brain may affect the rate of tumor growth, with gliomas developing slowly in the deep gray matter but showing increased proliferation in the lobar white matter (Ramnarayan et al., 2007; Adamson et al., 2010).

Environmental/occupational exposure and DNA damage are potential neurocarcinogenic factors (De Roos et al., 2003; Pinarbasi et al., 2005), which increases the importance of glutathione S-transferase (GST) systems for detoxification of exogenous and endogenous substances (Pinarbasi et al., 2005; Belpomme et al., 2007). Interestingly, under similar conditions, individuals respond differently to environmental carcinogen exposure and present distinct genetic susceptibility to cancer; the age-adjusted incidence of brain tumors is high in developed industrial countries (Ohgaki and Kleihues, 2005).

The human GSTs possess enzymatic and non-enzymatic functions and are involved in phase II metabolism, stress response, cell proliferation, apoptosis, oncogenesis, tumor progression, and drug resistance (Lo and Ali-Osman, 2007). GST isoenzymes catalyze the conjugation of reactive electrophiles generated during phase I detoxification and scavenging of free radicals created by radiation (Kilburn et al., 2010) forming water-soluble compounds easily removed by the body, including polycyclic aromatic hydrocarbon carcinogens (Pinarbasi et al., 2005; Belpomme et al., 2007; Kilburn et al., 2010). Based on sequence homology and immunological cross-reactivity, human GSTs have been grouped into 8 families: alpha, mu, theta, pi, zeta, sigma, kappa, and omega (Hayes et al., 2005).

Homozygous deletion of GSTM1 and GSTT1 genes eliminate GSTM1 and GSTT1 functional enzyme production (Hayes et al., 2005; Pinarbasi et al., 2005). A high percentage of American Caucasians (50%) possess the GSTM1 null genotype, whereas only 15% lack
GSTT1, which has been associated with increased susceptibility to meningioma (De Roos et al., 2003; Lai et al., 2005; Pinarbasi et al., 2005; Schwartzbaum et al., 2007) but not esophageal cancer in Whites and Asians; neither is associated with esophageal squamous cell carcinoma in Brazilian subjects (Lee et al., 2005). In contrast, lack of GSTM1 is associated in some groups with increased risk of solid tumors in the colon (Ebrahimkhani et al., 2012); bladder (Engel et al., 2002), lung (Carlsten et al., 2008), and stomach (Chen et al., 2010; Qiu et al., 2011). Studies correlating GST polymorphisms and risk of adult brain tumor have produced inconsistent results, indicating the need to establish a putative association with malignant gliomas, the most frequent and aggressive adult brain tumor.

Perillyl alcohol (POH), a dietary monoterpene found in a variety of plants, suppresses post-translational isoprenylation of the Ras small GTPase superfamily of proteins that stimulate tumor-associated angiogenesis and NFκB signaling (Holstein and Hohl, 2003; da Fonseca et al., 2008a; Chaudhary et al., 2009). This study compared the genotypic profile of GSTM1 and GSTT1 in a group of Brazilian patients with malignant glioma and a control group of healthy individuals living in the same geographical region. The aim was to establish whether GSTM1 and GSTT1 polymorphisms influence survival and response to intranasal administration of POH.

**MATERIAL AND METHODS**

This case-control study included a group of patients with malignant gliomas enrolled in a Phase I/II clinical trial to assess the efficacy of intranasal administration of the monoterpene POH. The cohort consisted of 95 patients (52 men and 43 women) with recurrent malignant glioma, mean age 53.4 years (range 19 to 83). Diagnosis and histological classification of malignant glioma based upon WHO criteria (Louis et al., 2007) indicated the following distribution: glioblastoma multiforme (GBM; N = 71), anaplastic astrocytoma (AA; N = 13) and oligodendroglioma (OD; N = 11). All patients were attending the outpatient Neurosurgical Unit of Antonio Pedro University Hospital. The control subjects included 100 healthy volunteers (36 men and 64 women), mean age 42.6 years (range 20 to 97). Demographic characteristics of patients and controls are shown in Table 1. Control subject eligibility was assessed by a detailed questionnaire; only subjects with no familial history of brain tumor, infectious disease, or use of anti-inflammatory medication were included. The Hospital Medical Research Ethics Committee and the Brazilian Ministry of Health (CONEP 25000.009267/2004-25) approved the study, which complies with the principles laid down in the Declaration of Helsinki.

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
<th>Control subjects (N = 100)</th>
<th>Glioma patients (N = 95)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (%)</td>
<td>36 (36%)</td>
<td>52 (55%)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>64 (64%)</td>
<td>43 (45%)</td>
</tr>
<tr>
<td>Mean age (range) years</td>
<td>42.6 (20-97)</td>
<td>53.4 (19-83)</td>
</tr>
<tr>
<td>Glioblastoma (%)</td>
<td>0</td>
<td>71 (75%)</td>
</tr>
<tr>
<td>Astrocytoma (%)</td>
<td>0</td>
<td>13 (14%)</td>
</tr>
<tr>
<td>Oligodendroglioma (%)</td>
<td>0</td>
<td>11 (11%)</td>
</tr>
</tbody>
</table>

Patients were stratified into groups according to clinical presentation (primary or secondary GBM), classification (GBM, AA, OD), and tumor location (deep gray matter or lobar regions) confirmed by computer tomography and/or magnetic resonance imaging. Patients re-
ceived 55 mg POH 4 times daily by intranasal administration (inhalation) for a total dose of 220 mg/day. Intranasal delivery is a suitable route for efficient and rapid delivery of small molecules with molecular weight less than 1 kDa (McMartin et al., 1987). The monoterpene POH is 152.8 Da and is hydrophobic, which allows it to cross the blood-brain barrier and reach the central nervous system, eliminating systemic losses and reducing side effects (da Fonseca et al., 2008b).

Circulating DNA was extracted from blood plasma or serum (Fleischhacker and Schmidt, 2007; Cabral et al., 2010) with the QIAamp DNA Blood® kit according to manufacturer protocols. Control DNA samples were extracted from cells from the oral mucosa and treated with 0.3 μg/μL proteinase K at 60°C for 2 h. Thereafter, an equal volume of 1:1 (v/v) phenol:chloroform was added, followed by vigorous shaking and centrifugation. The aqueous phase was separated DNA precipitated with 2 volumes of absolute ethanol at -20°C overnight. After centrifugation, pellets were washed with 70% ethanol and the DNA was resuspended in Milli-Q water. GSTM1 and GSTT1 genotypes were detected by multiplex polymerase chain reaction (PCR) with the p53 gene (exon 5) as an internal control. The following primers were used: GSTM1, 5'-GAACCTCCCTGAAAAGCTAAAGC-3' and 5'-GTGGGCTCAATATACGGTGG-3'; GSTT1, 5'-TTCCTTACTGGTCCTCACATCTC-3' and 5'-TCACCGGATCATGGCCAGCA-3'; p53 (exon 5), 5'-GCAACCAGCCCTGTCTGTCTCTCA-3' and 5'-GGAATTCTGTGATGTGAGCTCAATGAC-3'. The amplification conditions included denaturation at 94°C for 5 min followed by 30 cycles of 95°C for 30 s, 64°C for 1 min, 72°C for 1 min. The final extension was at 72°C for 5 min. After amplification, PCR products were separated by 10% polyacrylamide gel electrophoresis and visualized with silver nitrate. Gel staining was performed as follows: an initial step of DNA fixation using a 10% ethanol and 0.37% acetic acid solution for 10 min, a step of impregnation by 0.2% silver nitrate solution for 10 min followed by a rinse with distilled water for 30 s, and a final step with 3% NaOH and 0.4% formaldehyde solution until the DNA bands were clearly visible. The initial solution was used to stop the reaction. The SPSS 15.0 software was used for data analysis. The two-tailed Fisher exact test and odds ratio were used to compare groups (patients and control subjects) in the context of GSTM1 and GSTT1 deletion, and to determine the association between GSTM1 and GSTT1 genotypes with age, gender, and tumor type. The Williams G-test for independent samples was used to assess the correlation between GSTM1 and GSTT1 genotypes and median survival, and to correlate tumor location with survival. The Kaplan-Meier curves were used to analyze the association of GSTM1/GSTT1 genotypes and tumor location with survival.

RESULTS

Subjects (95 patients with malignant glioma and 100 controls) were genotyped for GSTM1 and GSTT1. The mean ages of patients and controls were 53.36 ± 16.0 and 42.6 ± 20.2, respectively. There was no statistically significant difference in familial cancer history between groups. Seventy-one glioma patients (75%) had at least 1 clinical and image recurrence event from primary and 24 (25%) from secondary GBM. Mean age significantly differed (P < 0.01) between AA (41.4 ± 16.62) and primary GBM (55.03 ± 15.07), but not anaplastic OD (52.4 ± 5.51), confirmed by the Dunn multiple comparison test.

A representative gel image (Figure 1) shows GSTM1 and GSTT1 genes as 220- and 450-bp bands; p53 (exon 5) appears as a 274-bp band. Genotype frequencies are shown in Table 2. GSTM1 deletion occurred in 42 (44%) patients and 54 (54%) control subjects (P =
GSTT1 deletion was detected in 11 (11.5%) patients and 36 (36%) control subjects, a statistically significant difference: OR = 0.23; 95%CI = 0.11-0.49; P = 0.00009. Genotype frequencies stratified by histology revealed a high GSTT1 frequency in patients with primary GBM (91.5%) and secondary GBM derived from anaplastic OD (90.9%), but not in those with AA (69.2%). Patients with AA carried the GSTM1 (76.9%) allele more frequently than those with primary GBM (50.7%) or anaplastic OD (63.6%).

Figure 1. Multiplex PCR of GSTM1 and GSTT1 genes and Tp53 exon 5. Representative electrophoresis shows GSTM1 and GSTT1 genes as 220- and 450-bp bands, respectively, and p53 (exon 5) as a 274-bp band. Lane 1 = 100-bp ladder; lanes 2, 4, 5, 6, 7, 8, and 10 = without deletion of GSTT1 or GSTM1; lane 3 = GSTT1 and GSTM1 deletion; lane 9 = GSTM1 deletion.

Table 2. Frequencies of GSTM1 and GSTT1 polymorphisms in patients and controls.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Control subjects (N = 100)</th>
<th>Glioma patients (N = 95)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM1 present (%)</td>
<td>46 (46%)</td>
<td>53 (56%)</td>
</tr>
<tr>
<td>GSTM1 nul (%)</td>
<td>54 (54%)</td>
<td>42 (44%)*</td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>1.00</td>
<td>0.67 (0.38-1.18)</td>
</tr>
<tr>
<td>GSTM1 present (%)</td>
<td>64 (64%)</td>
<td>84 (88%)*</td>
</tr>
<tr>
<td>GSTM1 nul (%)</td>
<td>36 (36%)</td>
<td>11 (11.5%)**</td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>1.00</td>
<td>0.23 (0.11-0.49)</td>
</tr>
<tr>
<td>GSTM1/T1 present (%)</td>
<td>30 (30%)</td>
<td>45 (47%)</td>
</tr>
<tr>
<td>GSTM1/T1 nul (%)</td>
<td>20 (20%)</td>
<td>3 (3%)***</td>
</tr>
<tr>
<td>Odds ratio (95% CI)</td>
<td>1.00</td>
<td>0.10 (0.02-0.36)</td>
</tr>
</tbody>
</table>

*P = not significant, **P = 0.00009, ***P = 0.000085.

Survival by GSTM1 and GSTT1 genotypes was determined by Kaplan-Meier curves, shown in Figure 2 (Lai et al., 2005; Kilburn et al., 2010). Patients without GSTM1 but with the GSTT1 deletion had a greater survival rate; patients with GSTM1 deletion had a median survival of 23 weeks, and those without the deletion survived 31.3 weeks (P = 0.86). Patients with the GSTT1 deletion presented a median survival of 18.7 weeks in comparison with 28.4 weeks in...
those without the deletion ($P = 0.0001$). The influence of anatomical tumor location on survival rate was also investigated (Figure 3). Tumor location was available for 44 patients; of these, 29 had lobar tumors with median survival of 33 weeks. Patients with tumoral lesions in deep regions (15/44) had a median survival of 39 weeks, a statistically significant difference ($P = 0.03$).

Figure 2. Survival of glioma patients under POH treatment stratified by genotypes. The Kaplan-Meier graphs show survival rates of patients according to $GSTM1$ and $GSTT1$ genotypes.
DISCUSSION

Malignant gliomas are biologically aggressive brain tumors associated with high morbidity and mortality. A meta-analysis of publications from different populations including 1630 cases of glioma and 7151 controls did not find an association between \textit{GSTM1}, \textit{GSTT1}, and \textit{GSTP1} variants and the risk of glioma (Lai et al., 2005; Kilburn et al., 2010). Moreover, \textit{GSTs} show substantial variations in frequencies between ethnic groups and different geographical environmental exposures (Di Pietro et al., 2010), which may explain the divergent results in Brazilians, who form an ethnically heterogeneous population (Carvalho-Silva et al., 2001). It was therefore important to assess polymorphisms in the phase I detoxification enzymes responsible for glutathione conjugation of alkylators and scavenging of free radicals in a population of patients with malignant glioma enrolled in a Phase I/II clinical trial of POH and who had failed multimodal therapy including surgery, radiation, and chemotherapy (da Fonseca et al., 2008a).

The frequency of \textit{GSTM1} deletion in Brazilian adults with malignant gliomas differs from other types of epithelial tumors (Engel et al., 2002; Carlsten et al., 2008; Chen et al., 2010) that show a positive association between \textit{GSTM1} deletion and tumor
development. In addition, a study of 78 patients and 374 controls did not reveal an association between GST polymorphisms and primary glioma risk in Brazilians (Coutinho et al., 2010). In this study, GSTM1 deletion was not significantly correlated (P = 0.19) with the occurrence of malignant glioma in adult Brazilians. GSTM1 deletion was observed in 44% of glioma patients and 54% of healthy controls. This result is consistent with a North American study that found a similar association between GSTM1 homozygous deletion and malignant gliomas (53%, OR = 1.1; 95%CI = 0.7-1.7) in 158 patients and 157 controls (Wiencke et al., 1997). Conversely, a significant decrease in the frequency of GSTM1 deletion was observed in pediatric patients with high-grade astrocytoma, but not in adults (Ezer et al., 2002; De Roos et al., 2003; Okcu et al., 2004; Lai et al., 2005; Diedrich et al., 2006; Kilburn et al., 2010).

We also observed GSTT1 deletion in only 11.5% of patients with malignant gliomas, versus 36% in healthy controls. This highly significant (P = 0.00009) difference suggests a negative association between GSTT1 gene deletion and the occurrence of malignant gliomas in the Brazilian population in this study. Similarly, GSTT1 deletion has been reported in a low percentage (13 to 16%) of patients with brain tumors, mostly malignant gliomas (Ezer et al., 2002; Okcu et al., 2004). Interestingly, a study conducted in Rio de Janeiro observed GSTT1 deletion in 25.4% of 591 healthy volunteers with no history of cancer (Rossini et al., 2002), although GSTT1 deletion may be associated with increased risk of meningioma (Lai et al., 2005).

Genetic polymorphisms in the GST family were associated with survival and toxicity secondary to chemotherapy with nitrosourea alkylating agents in a large group of American Caucasian patients with primary malignant glioma (Okcu et al., 2004; Diedrich et al., 2006). In the present study, GSTM1 or GSTT1 deletion also correlated with reduced survival in glioma patients. Indeed, patients with a GSTM1 deletion had an 8-week reduction in median survival compared to patients without the deletion. Glioma patients with the GSTT1 deletion survived about 10 weeks less than did patients without the deletion.

Established prognostic factors such as patient age, tumor histology, extent of surgical resection, and Karnofsky score often fail to predict survival (Gilbert et al., 2010; Weller et al., 2012). Although being unable to determine the level and localization of GSTP1 protein expression in tumors is considered an important variable associated with survival of malignant glioma patients (Okcu et al., 2004; Diedrich et al., 2006), there is a significant difference (P = 0.03) in survival of patients with lobar gliomas (33 weeks) in comparison to patients with deeply located tumors (39 weeks). This is consistent with previous findings that patients with deep gray matter glioma may survive longer than those with tumors in a lobar location (Ramnayaran et al., 2007). The present results partly explain previous data (da Fonseca et al., 2011) showing that tumor location and polymorphisms of GSTM1 and GSTT1 may exert additional influence on the survival of patients with malignant gliomas. It is important to conduct larger studies to confirm whether GST genotypes that encode high-activity enzymes influence POH detoxification, which may be an independent predictor of outcome and response to POH-based therapy.

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